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The Potential Role of Epstein-Barr Virus Nuclear Antigen-1 (EBNA-1) in Multiple Sclerosis

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Abstract

Background Multiple sclerosis (MS) is a disease affecting the central nervous system (CNS), with inflammation and demyelination of nerves, eventually resulting in nerve damage and disabilities. The risk of developing MS enlarged by infectious mononucleosis, which is caused by delayed primary infection by Epstein-Barr Virus (EBV). Early reports consistently demonstrated an increased antibody response in MS patients versus healthy subjects towards different EBV antigens, including the EBV nuclear antigen-1 (EBNA-1). In Iraq, number of researchers found that the EBV have a possible role at some point in the course of the disease, others reported that the EBV pathogenesis have an important role in the triggering of MS disease.

Objective To compare the sero-prevalence of EBV nuclear antigen-1 antibody (EBNA-1 IgG) among the Iraqi MS patients and controls and find out whether there is a relation between disease severity and EBNA-1 IgG titer.

Methods This case-control study conducted on 120 MS patients aged between 13-42 years, and 120 apparently healthy age- and sex-matched volunteers as controls. Three ml of whole blood were collected from all MS patients and controls and put in gel tubes. The blood samples were centrifuged at 5000 RPM for 5 minutes to get serum from the gel tubes. Serum was preserved in (-20°C), and then used for enzyme-linked immunosorbent assay (ELISA). The ELISA study was performed using ELISAs kits (Abnova /Taiwan) for EBNA-1 IgG antibodies measurement.

Results EBNA-1 IgG was positive in 51.7% (62/120) of MS patients and 39.2% (47/120) of controls, (P=0.035). The median of EBNA-1 IgG level of MS patients and controls were 81.08 U/ml, and 67.73 U/ml, respectively (P=0.043). And EBNA-1IgG was significantly higher in younger age groups. Patients with the first-line and second-line treatment showed no significant differences in EBNA-1 IgG levels, while the median level in patients without treatment (newly diagnosed) and those who were at early years of the disease was higher.

Conclusion EBNA-1 antibody could play a triggering role in MS because it is significantly higher in MS patients than in controls, especially at younger age groups, early stages of the disease and in female patients.

Keywords Multiple sclerosis, EBV, EBNA-1 IgG, ELISA

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List of abbreviations: EBV = Epstein-Barr virus, EBNA-1 IgG = Epstein-Barr virus nuclear antigen-1 antibody, ELISA = Enzyme-linked immunosorbent assay, IgG = Immunoglobulin G, MRI = Magnetic resonance imaging, TMB = Tetramethylbenzene

Introduction

Multiple sclerosis (MS) is a disease affecting the central nervous system (CNS), with inflammation and demyelination of nerves, eventually resulting in

nerve damage and disabilities [1]. Young adults are most commonly affected by MS while women are more susceptible and men have worse progression [1,2]. It is assumed that both genetic and environments factors are effective on this disease [3].

Epstein-Barr virus (EBV) is also referred to as the human gamma herpesvirus, the disease is asymptomatic in childhood, but it becomes symptomatic in adolescents. The frequency of MS is nearly 15 times higher in early childhood with EBV and about 30 times higher among adolescent and later-life patients who have EBV infections [4]. An increased antibody response has been seen in MS patients versus healthy subjects towards different EBV antigens, including the EBV nuclear antigen-1 (EBNA-1) [5].

EBNA-1 is expressed in all actively dividing EBV-infected cells and is responsible for fusion of the viral episome to the mitotic cellular DNA, confirming duplication and transport of virus genome to all daughter cells [6]. EBNA-1, the essential EBV antigen for virus latency, makes up a principal antigen for both cell-mediated and humoral immune responses against the virus, and in MS the deregulation of immunity specific for EBV has been reported principally for this antigen [7]. Increased levels of EBNA-1-specific antibody responses may also predict conversion from clinically isolated syndrome (CIS) to MS [8].

Antibody responses to EBNA-1 reach their highest titer during convalescence from infectious mononucleosis. Notably, epidemiological studies reported that the increase in EBNA1-specific IgG titers precede the onset of MS in various populations [9]. Ascherio et al. [9] found that IgG specific for EBV nuclear antigens were significantly elevated in plasma collected before the onset of MS and did not change significantly after MS onset.

To the best of our knowledge, there are two previous studies regarding the seroprevalence of EBNA-1 IgG in Iraq, Bakir et al. in 2017 showed significantly higher level in MS patients than the healthy controls and he was revealed the possible role of EBV during the course of MS autoimmune disease via antibody EBNA-1 IgG seropositivity and correlated with type of attack or relapse [10], Abd Al Kareem and Abd in 2020 reported that mean serum level was significantly higher in female patients than in the healthy controls and they revealed that the pathogenesis of EBV has crucial role in initiation MS disease in females patients [11]. The objective of this study is to find out any correlation between MS development and the incidence and levels of anti- EBNA-1 IgG.

Methods

A case-control study conducted on 120 patients with MS aged between 13-42 years from November 2020 to June 2021. Blood samples were obtained from MS patients in the MS clinic in the Baghdad Teaching Hospital of Medical City Complex, and 120 controls were obviously healthy age and sex-matched volunteers collected from the blood donation centers. Informed consents were obtained from all subject before sampling. This study was approved by the Institutional Review Board of the College of Medicine, Al-Nahrain University (No.20200980 on 17/11/2020). The study was conducted in the labs of Microbiology Department at the College of Medicine -Al-Nahrain University. Patients' clinical parameters included: the duration of MS, number of relapses, the line of treatment and type of treatment. Exclusion criteria: Patients on rituximab therapy. Three ml of whole blood were collected from all MS patients and controls and put in gel tubes. The blood samples were centrifuged at 5000 RPM for 5 minutes to get serum from the gel tubes. serum was preserved in (-20^{o}C), and then used for enzyme-linked immunosorbent assay (ELISA).

The ELISA study was performed using ELISAs kits (Abnova/Taiwan) for EBNA-1 IgG antibodies measurement depended on the binding of antibodies in the sample with EBNA-1 antigen that coat the wells of ELISA plate and the antibodies being in complexes with antigen are later recognized by animal anti-human IgG

antibodies labelled with horseradish peroxidase. The labelled antibodies are revealed by an enzymatic reaction with a chromogenic substrate. Sensitivity of the test was 100%, and Specificity was 96.4%.

For quantitative evaluation the sample antibody titers in artificial units (AU/mL) were computed as follows: 1. A calibration curve was constructed by plotting the units of Standards (x-axis) to absorbance of Standard (y-axis). 2. The place where the absorbance of tested samples intersect calibration curve were found and the corresponding values (AU/mL) on the axis x were found.

Statistical Analysis

Statistical package for social sciences (SPSS) Inc., Chicago,IL, USA, Version 21 was used for statistical analysis; categorical data were formulated as count and percentages, and Chi-square test was used to describe the association of these data. Numerical data were described as the median with percentile. And independent sample t-test was used for comparison between the two groups. The lower level of statistically significant difference was regarded as ≤ 0.05.

Results

Among the 120 MS patients; 48 (40%) were males and 72 (60%) were females, there was no significant difference in sex distribution between patients and controls (P= 0.447). The median age of MS patients was 32 years and also there was no statistically significant difference between the age of the MS patients and controls for the different age groups indicating that they were of a comparable age (P= 0.465). According to the duration of MS disease in the studied patients; 75% of MS patients (90 out of 120 patients) had MS for more than 2 years, most of MS patients (76.67%) 92 out of 120 were having a low number of relapses of less than 3 relapses, and the lower percentage of patients (23.33%) 28 out of 120 were having a higher number of relapses of more than 3 relapses.

The treatments used by MS patients included Avonex (β-interferon-1a), Betaferon (β-interferon-1b), Rebif (β-interferon-1a), Gilenya (Fingolimod), Natalizumab (Tysabri), and the last group were without treatment, most of them were newly diagnosed. The largest group of the patients were on Natalizumab regimen (40.83%), and the smallest group of the patients were on Avonex (5%). Large number of MS patients (48.33%) treated with the second line therapy that included (Natalizumab or Gilenya).

The results of ELISA showed that EBNA-1 IgG was positive in 51.7% (62/120) of MS patients and 39.2% (47/120) of controls, The median of EBNA-1 IgG level of MS patients were 81.08 U/ml (Percentile 25=57.88, Percentile 75=101.40), and of control were 67.73 U/ml (Percentile 25=54.62, Percentile 75=103.93), (Table 1).

Table 1. Comparison of EBNA-1 IgG index values between MS patients and controls

EBNA-1 IgG	MS		Control		P-value
Negative	58	48.3%	73	60.8%	0.035*
Positive	62	51.7%	47	39.2%	
Median	81.08		67.73		
Percentile 25	57.88		54.62		0.043*
Percentile 75	101.40		103.93		

This study showed that the rate of seropositivity and median of EBNA-1 IgG level of the MS patients were significantly higher than the controls in relation to the age groups:

(<21 years) ((P= 0.050, 0.006), and (21-30) (P= 0.003, <0.001) respectively; whereas in the remaining age groups (31-40), (>40), there was no significant differences in the median level and seropositivity rate of EBNA-1 IgG between MS patients and controls, (Table 2).

Table 2. EBNA-1 IgG results in MS patients and controls in relation to age groups

Age range	EBNA-1 IgG	MS		Control		P value
<21 years	Negative	6	42.9%	10	83.3%	0.050*
	Positive	8	57.1%	2	16.7%	
	Median	80.85		54.42		
	Percentile 25	55.23		45.73		0.006*
	Percentile 75	94.58		63.84		
21-30 years	Negative	20	42.6%	30	75.0%	0.003*
	Positive	27	57.4%	10	25.0%	
	Median	94.83		63.59		
	Percentile 25	55.63		54.92		<0.001**
	Percentile 75	102.32		79.94		
31-40 years	Negative	27	55.1%	30	49.2%	0.569
	Positive	22	44.9%	31	50.8%	
	Median	73.62		80.62		
	Percentile 25	59.32		56.24		0.755
	Percentile 75	104.99		112.46		
>40 years	Negative	5	50.0%	3	42.9%	0.995
	Positive	5	50.0%	4	57.1%	
	Median	86.66		81.31		
	Percentile 25	61.81		59.53		0.894
	Percentile 75	99.43		126.14		

**P value highly significant <0.001, *P value is significant <0.05

The median of EBNA-1 IgG titer was significantly higher in females compared to the males (P= 0.004) (Table 3), in addition, females have higher percentage of IgG seropositivity than males (59.5 % in females versus 39.1% in males), as shown in the table (3).

Table 3. ENBA-1 IgG result in relation to sex among MS patients and controls

EBNA-1 IgG	Study groups			
	MS		Control	
	Female	Male	Female	Male
Negative	30 (40.5%)	28 (60.9%)	44 (61.1%)	29 (60.4%)
Positive	44 (59.5%)	18 (39.1%)	28 (38.9%)	19 (39.6%)
Median	94.58	63.91	66.67	67.84
Percentile 25	64.12	53.81	53.40	55.84
Percentile 75	104.99	97.88	109.79	101.79
P value	0.004*		0.995	

The rate of seropositivity and median of EBNA-1 IgG is significantly higher in the patients who have the disease for 2 years or less than in the patients with a disease duration more than 2 years (p<0.001, P=0.029 respectively), (Table 4).

Table 4. The relation between EBNA-1 IgG serology and disease duration

EBNA-1 IgG	Disease duration		P value
	≤2 years	>2 years	
Negative	5 (16.67%)	53 (58.89%)	<0.001**
Positive	25 (83.33%)	37 (41.11%)	
Median	82.81	64.22	
Percentile 25	55.43	53.81	0.029*
Percentile 75	126.14	97.37	

**P value highly significant <0.001, *P value is significant <0.05

This study observed no statistically significant association of EBNA-1 IgG serology result with number of relapses (p= 0.812), as shown in table (5).

Results in table (6) illustrated that there was no statistically significant association of the EBNA-1 IgG results with line of treatment (p=0.549).

Table 5. The relation between EBNA-1 IgG serology and number of relapses

EBNA-1 IgG	Number of relapses		P value
	≤3 relapses	>3 relapses	
Negative	45 (48.91%)	13 (46.43%)	0.812
Positive	47 (51.09%)	15 (53.57%)	
Median	68.38	64.86	
Percentile 25	55.02	54.21	0.833
Percentile 75	107.59	96.56	

**P value highly significant <0.001, *P value is significant <0.05

Table 6. The relation between EBNA-1 IgG serology and line of treatment

EBNA-1 IgG	Line of treatment			P value
	No treatment	1st line	2nd line	
Negative	5 (41.67%)	22 (44.00%)	31 (53.45%)	0.549
Positive	7 (58.33%)	28 (56.00%)	27 (46.55%)	
Median	94.87	63.59	66.67	
Percentile 25	64.17	54.42	53.2	0.390
Percentile 75	144.13	101.26	102.32	

Discussion

MS is a CNS disease characterized by demyelination, inflammation, and neuronal destruction. Genetic and environmental factors are coupled with the danger of developing MS, other than the precise reason still remains unidentified. Among the well-recognized environmental risk factors in MS were EBV, smoking, and vitamin D deficiency. The risk of developing MS enlarged by infectious mononucleosis, which is caused by delayed primary infection by EBV. Potentially the EBV acts together with both genetic and additional environmental risk factors to amplify receptiveness to and severity of MS disease [12]. A number of studies communicate EBV with MS [13,14], while others locate no association [15,16]. One of the most consistent pieces of evidence is the finding of elevated antibody titers against EBNA-1 antigen in the blood, both pre- and post-onset of the disease [17].

Several hypotheses explain the mechanism of EBV involvement in MS pathogenesis. One of these is molecular mimicry hypothesis, whereby EBNA-1-specific T cells from MS patients are cross reactive to myelin antigen [18]. The presence of an antigen such as the myelin basic protein (MBP), peptide which is derived from the myelin sheaths surrounding an axon having a homology to EBV viral proteins. Myhr et al., have illustrated molecular mimicry of viral EBNA-1 to MBP that could prompt T cell autoimmunity to myelin sheaths. For this reason, one of the most relevant non-self-antigens that is thought to induce MS is EBNA-1(19).

In the current study, EBNA-1 IgG antibody was positive in 51.7% (62/121) of MS patients and 39.2% (47/121) of controls. To the best of our knowledge, there are two previous studies regarding the seroprevalence of EBNA-1 IgG in Iraq, the more recent study carried out by Abd Al Kareem and Abd in 2020 among the Iraqi female patients with MS who were admitted to Clinic of MS in Neuro-Science Hospital in Baghdad and they reported that mean serum level was significantly higher in female patients than in the healthy controls [11], another study also show significantly higher level in MS patients than the healthy controls (Bakir et al., 2017), which were conducted in Rizgary Teaching Hospital in Erbil, Iraq [10].

The significantly higher seropositivity of EBNA-1 IgG in the MS patients than in the controls is in accordance with other studies [20,21], however, other studies, (Banwell et al., 2007), which was carried out on children, failed to manifest any relationship between the virus and MS [22]. The median level of EBNA-1 IgG for MS patients and controls were 81.08 U/ml and 67.73 U/ml respectively, which was significantly higher in MS patients in the current study, as reported by other studies which have showed significantly higher IgG level in MS patients [10,11,23,24], while others found no significant difference [25,26].

Concerning the association between EBNA-1 IgG results and duration of MS disease, the current study revealed higher serum level and seropositivity of EBNA-1 IgG in MS patients during first two years of disease and this result is in accordance with other study [11], this is indicative of the role of the viral antigen in the early stages of the disease which could be the triggering factor of MS. And due to this triggering factor, the relationship linking EBNA-1 IgG titers and number of clinical relapses showed no statistically significant association as shown in the table (5), this result is close to what's mentioned by Bakir et al. in 2017 [10].

On the other hand, in this study, patients with the first-line and second-line treatment showed no significant differences in EBNA 1 IgG levels between each other, while the median level in patients without treatment were higher, this result is comparable to result of study carried out by Abd Al Kareem and Abd in 2020 [11], these patients who were recently diagnosed with MS had very high median IgG titer also supporting the possible triggering factor by the viral antigen.

In addition, the current study observed that median of EBNA-1 IgG titer was significantly higher in females compared to the males (Table 3), MS is a disease of females, the female to male ratio is about 2:1 [27]. In addition, females have higher percentage of IgG seropositivity than males (59.5% in females versus 39.1 in males), as shown in the table (3). Foroutan-Pajoohian et al. in 2018, also have

reported higher seropositivity of EBNA-1 IgG in MS females than males [28], the above data may possibly be correlated with the more robust immune response enhanced by estrogens compared to the immunosuppressive function of androgens. Females have a better humoral immune response than males, as manifested by higher titers of serum immunoglobulin, and a larger antibody response to a variety of antigens after immunization [29]. However, Bakir et al., in 2017, Kreft et al., in 2017 show no statistically significant difference of mean EBNA-1 IgG titer according to patients' sex [10,23].

The present study clarified statistically significant differences in EBNA-1 IgG titer and seropositivity between MS patients and controls among age groups (<20) and (20-30) years as compared to the controls. This result was somewhat supported by Kreft et al., in 2017 who found that age sampling was significantly correlated with EBNA-1 IgG titer [23].

The current study aimed to investigate the role of EBV in the MS pathogenesis, either as a triggering or initiating factor through the primary active infection, or have a role in initiating relapses through the reactivation from the latent state. Because of higher level and seropositivity in the younger age groups and in the patient who haven't start treatment yet (newly diagnosed) and because there was no significant association in EBNA-1 results with number of relapses. This result may indicate that the EBV could have an important triggering role in MS disease.

Results of EBNA-1 IgG antibodies indicate that the virus could have an indirect effect in development of MS mainly through cross-reacting EBNA-1 IgG antibodies which were significantly higher in MS patients as compared with control subjects. Therefore, it is possible to move towards the use of immune therapy to reduce autoimmunity due to the virus.

In conclusion, EBNA-1 could have an important triggering role of MS because of significantly higher levels both quantitatively and qualitatively in MS patients than in controls, especially at younger age groups, early stages of the disease (in those who haven't start treatment yet), and in the female sex.

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Author contribution

Ali: did the laboratory work and write the article. Dr. Al-Obaidi: Supervision of the study and final editing of the manuscript. Dr. Al-Mashta: consultant and examined the patients.

Conflict of interest

Authors declare that there is no conflict of interest.

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